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PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Coppens et al.
Serial No.: 08/898,736
Filed: July 23, 1997
Title: PROCESS FOR THE PREPARATION OF MALTED CEREALS
Group Art Unit: 1761
Examiner: C. Sherrer

SUPPLEMENTAL DECLARATION OF THEO COPPENS UNDER 37 CFR 1.132

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Dear Sir:

I, Theo Coppens, pursuant to 37 C.F.R. §1.132, declare as follows:

1. I am one of the inventors for the above-identified patent application.

2. In 1999, I asked Prof. C. Michiels, Professor of the Faculty of Agricultural and Applied Biological Sciences at Katholieke Universiteit Leuven in Belgium, to conduct the following experiments under my supervision to determine whether the medium and growth conditions described in Gyllang et al. would provide activated spores. Those experiments and their results were first reported in my Declaration signed on

July 9, 1999. A more detailed explanation of those experiments and their results is presented herewith.

Materials and Methods

3. Chemicals and Media. Peptone, Yeast Extract and Potato Dextrose Agar (PDA) were obtained from Unipath (Hampshire, United Kingdom). Dextrose was obtained from Merck-Belgolabo (Leuven, Belgium). Peptone, Yeast Extract and Dextrose medium was prepared according to Kaiser et al. (1994). Peptone (2% w/v), Yeast Extract (1% w/v) were dissolved in deionised water and sterilized at 121°C for 20 minutes. The pH of the obtained medium was 6.4.

4. Fungal Strains, Cultivation and Preparation of Culture Homogenate. The strains *Rhizopus oryzae* ATCC 9363, *Aspergillus fumigatus* CBS 148.89 and Aspergillus *amstelodami* VTTD-76035 were obtained from respectively the American Type Culture Collection (ATCC, Manassas, VA, USA), Centraalbureau voor Schimmelcultures (CBS, Baarn, The Netherlands) and VTT (Technical Research Centre of Finland, Espoo, Finland) Culture collections. The strains were grown on PDA at 28°C. Seven days old sporulating cultures on PDA served as the starting material for culturing the fungi as described by Gyllang et al. (1977). For each strain a loopfull of material taken from the seven days old sporulating culture on PDA was inoculated in a tissue culture flask containing 225 ml of Peptone, Yeast Extract and Dextrose medium. The culture was grown for 3 weeks at 20°C. After the cultivation period the entire culture was homogenized by vigorously shaking the content of the tissue culture flask.

5. Analysis of activation of the spores in the culture homogenate. Activated spores were defined as described in the current patent application as "being significantly more swollen than the dormant size, the size of the spores being increased by a factor preferably between 1.2 and 10 over the dormant spore size and/or having one or more germ tubes per spore." Three different samples of 0.2 ml of the culture homogenate were examined microscopically. Swelling of the spores was verified by measuring the spores at a magnification of 1250x by means of an eyepiece graticule micrometer. Activation was reported as a percentage of the spore population, determined by microscopic count. Therefore, the spore population was quantified by means of a Thoma counting chamber at magnification of 320x (Carl Zeiss, Jena, Germany). At least 100 spores per sample were evaluated.

Results

6. Analysis of spore activation. The dormant size of various fungal spores is described by Pitt and Hocking (1997). According to this reference, the sporangiospores of *Rhizopus oryzae* are of variable shape, ellipsoidal to broadly fusiform or irregularly angular, commonly 5.0 - 8.0 µm long, the condiospores of *Aspergillus amstelodami* are spherical to subspheroidal with 4.0 - 5.0 µm diameter; the condiospores of *Aspergillus fumigatus* are spherical to subspheroidal with 2.5 - 3.0 µm diameter. Our own observations of dormant spores of the three tested strains were in agreement with the description given by Pitt and Hocking (1997). Accordingly, we defined activated spores of *Rhizopus oryzae* ATCC 9363, *Aspergillus fumigatus* CBS 148.89 and *Aspergillus amstelodami* VTT D-76035 as having respectively a size of more than 9.6 µm,

6.0 μm and 3.6 μm and/or one or more germ tubes per spore. Figure 1 shows some microphotographs of dormant, swollen and activated spores of *Rhizopus oryzae* ATCC 9363.

Figure 1. Activated spores obtained by treatment as described in the current patent application (magnification 720x): A. dormant spores; B. swollen spores with one activated (Ac) spore, i.e. significantly more swollen than the dormant size; C. activated spores significantly more swollen than the dormant size and having one or more germ tubes per spore.

The results of the analysis of spore activation in the culture homogenates immediately after homogenisation (0 time) under the procedure of Gyllang et al. (1977) are presented in Table I.

Table I. Spore Activation at 0 Time in the Culture Homogenates.

	% spores activated
Rhizopus oryzae ATCC 9363	0
Aspergillus fumigatus CBS 148.89	0
Aspergillus amstelodami VTT D-76035	0

Further activation of spores in the culture homogenates was analyzed after 6 hours incubation of the culture homogenates at 20°C or 42°C, although this deviates from the procedure of Gyllang et al. (1977). In this procedure no incubation period is prescribed between preparation of the homogenate and inoculation of the barley. The results are presented in Table II.

Table II. Spore Activation After 6 Hours Incubation in the Culture Homogenates.

	% spores activated	
	Incubation at 20°C	Incubation at 42°C
Rhizopus oryzae ATCC 9363	0	0
Aspergillus fumigatus CBS 148.89	0	0
Aspergillus amstelodami VTT D-76035	3	0

In contrast, treatment of *Rhizopus oryzae* ATCC 9363 spores as described in the current patent application resulted in a high level of activation of the spore as more than 90% of the spores had a size of more than 9.6 μm and/or had one or more germ tubes per spore.

7. Conclusions. Culture homogenates of *Rhizopus oryzae* ATCC 9363, *Aspergillus fumigatus* CBS 148.89 and *Aspergillus amstelodami* VTT D-76035 prepared according to Gyllang et al. (1977) do not contain activated spores. This experiment shows that successful activation depends on incubation of dormant spores for a sufficient time at a suitable temperature and in a suitable medium. In the spore suspension as prepared by Gyllang *et al.* (1977) the medium is an exhausted growth medium that does not provide the suitable conditions for spore activation, and the spores are not incubated for a sufficient time at a suitable temperature.

8. Abbreviation used. PDA, Potato Dextrose Agar; ATCC; American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures; VTT, Technical Research Centre of Finland; Ac, activated.

9. References.

Gyllang, H., Satmark, L. and Martinson, E., The influence of some fungi on malt quality, EBC Proceedings of the 16th Congress, 1977.

Kaiser, C., Michaelis, S. and Mitchell, A., Methods in yeast genetics, Appendix A, p. 207, Cold Spring Harbor Laboratory Press, New York, USA, 1994.

Pitt, J.I. and Hocking, A.D. Fungi and food spoilage, second edition, Blackie Academic & Professional, London, UK, 1997.

The undersigned, being warned that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. §1001) and may jeopardize the validity of the application or any patent issuing thereon, hereby declares that the above statements made of my own knowledge are true and that all statements made on information and belief are believed to be true.

Date: 03/03/2000

Theo Coppens